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Simultaneous analysis of various mutations on the 21-hydroxylase gene by multi-allele specific amplification and capillary gel electrophoresis

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Abstract

A detailed study is presented on the detection of various known point mutations using polymerase chain reaction (PCR) based multi-allele specific amplification (MASA) in conjunction with capillary gel electrophoresis (CGE) separation. The resulting PCR products, corresponding to the individual mutations, are labeled with ethidium bromide during CGE separation, and detected by laser-induced fluorescence. MASA proved to be a novel, fast and cost-effective method for simultaneous analysis of multiple known mutation sites, employing more than one allele specific primers in a single PCR reaction. It results in coexisting amplification of numerous DNA fragments differing in size, which are subsequently separated by CGE. In the present study, several point mutations were analyzed simultaneously by MASA–CGE on the 21-hydroxylase gene of a patient with congenital adrenal hyperplasia. © 1998 Published by Elsevier Science B.V.

Keywords: Genes; Multi-allele specific amplification; DNA

1. Introduction

Detection of disease-causing mutations has a growing importance in medical diagnosis [1]. Many diseases are caused by a single base pair change (point mutation) in the human genom, altering the active conformation of an important enzyme in the human body. One example is the deficiency of 21-steroid-hydroxylase, causing congenital adrenal hyperplasia [2]. Screening of mutations in the CYP 21 gene has a great medical importance, making possible prenatal diagnosis of this disease [3]. Allele specific amplification (ASA) is one of the methods, suitable for detection of point mutations using polymerase chain reaction (PCR). The principle of ASA is based on the fact that under certain conditions the Taq DNA polymerase, with no 3′ exonuclease activity, is unable to start, and therefore, produce a PCR product in case of mismatch between the 3′ end of the primer and the template [4]. Using this principle, one can detect any point mutation by designing a primer, which anneals with its 3′ end exactly to the site of the mutation of interest (Fig. 1). If the mutation is present in the human gene examined, an allele specific PCR fragment is formed (upper panel). If the gene does not contain this particular mutation, the enzyme does not start the reaction, thus no PCR product is formed (lower panel). As a control of this experimental arrangement, a normal (wild type) primer can be used that anneals to the non-mutated sequence only, thus, does not match with the sequence having the mutation.

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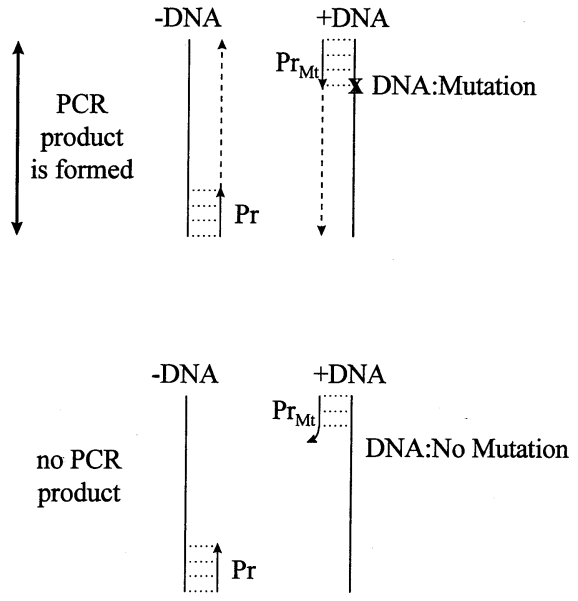

Fig. 1. Theoretical basis of allele specific amplification (ASA) mediated identification of the presence or absence of a single base-pair mutation. Single base-pair mutations can be identified by specifically designed primers (Pr_{Mt}), having the complementary sequence to the mutated DNA in its 3′ end. PCR amplification takes place only if the mutation is present in the DNA (upper panel). In the instance of normal DNA, not having this specific mutation, the 3′ end of Pr_{Mt} does not match the template strand, resulting in lack of PCR amplification (lower panel).

Capillary gel electrophoresis (CGE) has been recently emerging as a high resolution, rapid separation tool for the analysis of double stranded DNA molecules [5–10]. In conjunction with laser-induced fluorescent (LIF) detection, extremely high sensitivity can be obtained [11]. Besides of the commonly used pre-separation labeled dsDNA molecules, applications have been shown by using intercalator dyes such as ethidium bromide [12] to accommodate the 488 nm argon-ion or more recently the 532 nm Nd–YVO_4 laser. Using this methodology the single stranded PCR primer is unlabeled, only the dsDNA PCR fragments get labeled by intercalation with a fluorescent dye, such as ethidium bromide [13]. This complexation is mediated by having the appropriate amount of ethidium bromide in the gel–buffer system that intercalates with the dsDNA molecules as they migrate through the gel filled capillary column. The separated dsDNA–dye complexes are then detected in real time by the laser induced fluorescence detection system.

The extremely fast separation, inherent to CGE and the use of fluorescent intercalator dyes provided a rapid and highly efficient separation system with very sensitive detection. The goal of our study was to demonstrate that CGE can be applied as an effective high sensitivity and rapid separation tool for testing point mutations in human samples, by allele specific PCR in multiplex format.

2. Materials and methods

2.1. Chemicals

Tris, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS), EDTA and acrylamide all in electrophoresis grade were obtained from ICN (Costa Mesa, CA, USA). Linear polyacrylamide (4%) was polymerized outside of the separation capillary in 50 m*M* Tris-TAPS/2 m*M* EDTA buffer (pH 8.4) by the addition of 1 μl 10% ammonium persulfate and 1 μl *N,N,N′,N′*-tetramethylethylenediamine (TEMED) per ml reaction mixture. This pre-polymerized gel–buffer system was then filled into the coated capillary column before each run by a micro-syringe, applying positive pressure. The 8 cm effective length (12 cm total length) DB-1 coated capillary column (50 μm I.D.×375 μm O.D.) was used in the experiments (J&W, Folsom, CA, USA). The allele specific primers have been synthesized by the Agricultural Biological Center (Gödöllo, Hungary). The human DNA samples were the kind gift of Dr. J.Sólyom (2nd Department of Pediatrics, SOTE, Budapest, Hungary). The Taq DNA polymerase and the ΦX174 restriction digest fragment mixture were from Pharmacia Biotech (Uppsala, Sweden). All other chemicals were from Sigma (St. Louis, MO, USA).

2.2. Allele specific PCR amplification.

Genomic DNA was isolated from the blood of a patient with congenital adrenal hyperplasia, using phenol extraction followed by ethanol precipitation [14]. Conditions of the allele specific PCR were similar to Ref. [15]. Briefly: The genomic DNA was

first amplified to obtain a 2063 base pair (bp) fragment, using a carefully designed PCR primer set to avoid amplification of the pseudogen, that has almost identical sequence than the CYP 21 gene, except of an 8 bp deletion in Exon 3, causing a frameshift. This amplified 2063 bp fragment was used later as a template for re-PCR by the allele specific primers. A typical PCR reaction mixture contained 10 m*M* Tris-HCl (pH: 9), 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 5% glycerol, 200 μ*M* of each dNTP, 0.5 μ*M* of all primers used, 1 U of Taq DNA polymerase and 0.1 ng of target DNA or 1 μl of the first PCR product in a final volume of 20 μl. 30 cycles at 96°C for 1 min, 54°C for 30 s and 72°C for 3 min were performed in an Ericomp thermal cycler (San Diego, CA).

2.3. Instrumentation

The laboratory-made, laser induced fluorescence detection based capillary electrophoresis system employed a solid state frequency doubled Nd–YVO_4 laser (532 nm) excitation source and an avalanche photodiode for detection. Fig. 2 depicts the instrumentation setup. The 532 nm beam from the 5 mW green frequency doubled Nd–YVO_4 microlaser (1)(B&W Technology, Newark, DE, USA) was passed through a 6.3×microscope objective (2)(Melles Griot, Irvine, CA, USA) onto the detection window (3 mm) of the horizontally positioned fused-silica capillary column (3). Two microfuge vials (250 μl each) were used as buffer reservoirs (4), refilled with fresh gel–buffer after each run. The electric circuit was closed by means of platinum wires connected to a 30 kV EMCO (Sutter Creek, CA, USA) power supply (5). The emitted fluorescent light was collected in 90° angle, passed through a pinhole (7) (Melles Griot), sandwiched between two 8 mm aspheric lenses (6) with 0.5 NA (Geltech, Orlando, FL, USA), followed by a 630 nm (center wavelength 45 nm wide) bandpass filter (8)(Omega, Brattleboro, VT, USA) and another aspheric lens (9) similar to the one above (6), into a Hamamatsu avalanche photodiode (10) (C5460-01, Hamamatsu, El Cajon, CA, USA). The signal was collected with 10 Hz sampling rate by a microcontroller (11) (V-104, Tern, Davis, CA, USA) and acquired by a personal computer system (12). The collected data was evaluated by the Caesar 4.1 software package (CE Solutions, Long Branch, NJ, USA).

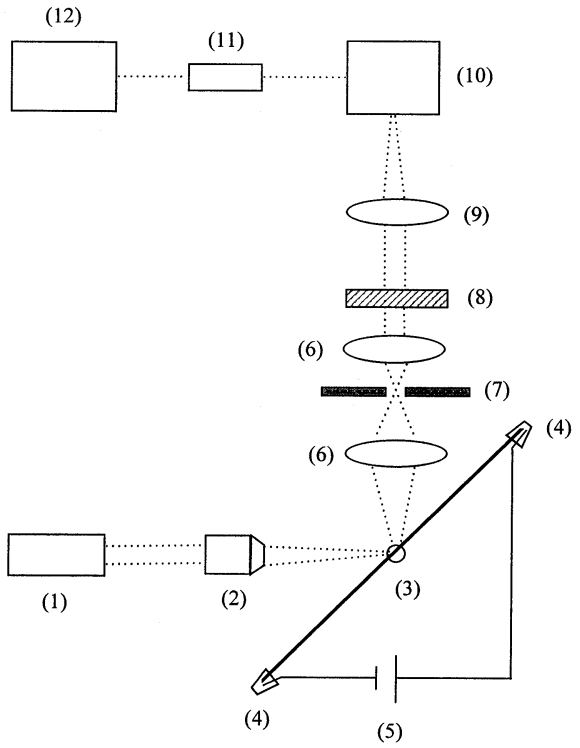

Fig. 2. Block diagram of the CE–LIF detection system. Details of the components are described in Section 2.

3. Results and discussion

3.1. Separation of standard ΦX 174 restriction fragment mixture

The in-house made capillary electrophoresis–laser induced fluorescent detection system was first evaluated by separating a standard dsDNA restriction fragment mixture of ΦX 174 Hae III digest. As Fig. 3 depicts, decent separation of all the 11 components were obtained within 2.5 min. The migration times of these fragments were used in the following experiments for PCR product chain length evaluation purpose. Please note that with this very high sepa-

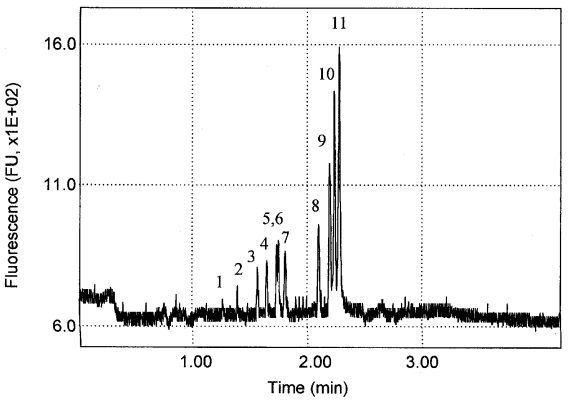

Fig. 3. Capillary gel electrophoresis separation of ΦX 174 HaeIII digest restriction fragment mixture. Peaks: (1) 72; (2) 118; (3) 194; (4) 234; (5) 271; (6) 281; (7) 310; (8) 603; (9) 872; (10) 1078; (11) 1353 bp. Conditions: Detection: LIF 532 nm/630 nm; Capillary: 8 cm (effective, 12 cm total) DB-1 capillary column (50 μm I.D.); Gel–buffer: 4% linear polyacrylamide gel in 50 m*M* Tris–TAPS, 2 m*M* EDTA (pH 8.3) buffer containing 0.5 μg ml^{-1} ethidium bromide; E=400 V cm^{-1}, I=18 μA, temp: 25°C; Electrokinetic injection: 600 V (10 s).

ration speed, the 271/281 bp fragments were only partially separated.

3.2. Multi-allele specific amplification (MASA)

A highly reliable method using simple allele specific amplification was described earlier by Wedell and Luthman [15] for clinical screening of single mutations that cause 21-steroid hydroxylase deficiency. The goal of our study was to increase the throughput of the identification of rare mutations by multiplexing several allele specific primers, thus, we introduced the method of multi-allele specific amplification (MASA). The carefully designed primers used in these experiments for allele specific amplification of the 21-hydroxylase gene (Table 1) were

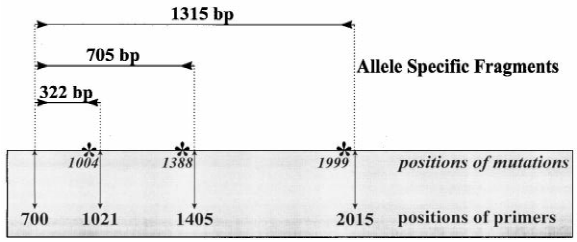

Fig. 4. Principle of multi-allele specific amplification (MASA).

applied in a specific combination in order to screen for more then one mutation in a multiplexed PCR reaction. When the three mutant primers (Table 1, column 4) were used in the multiplexed PCR reaction as sense primers, in conjunction with a single common antisense primer (Table 1, last row), PCR product formed only when any of the three mutations was present in the gene (Fig. 4). Please also note in Fig. 4, that the size of each generated fragments was characteristic of the mutation identified. Normal (wild type) sense primers (Table 1, column 3) could be also used in conjunction with the common antisense primer in a second PCR reaction mixture for control purpose. In this instance, allele specific fragments are formed that correspond to the normal (wild type) sequence only.

Fig. 5 shows the result of multi-allele specific amplification (MASA) in combination with capillary gel electrophoresis in case of a patient with congenital adrenal hyperplasia. When the mutant versions of the three allele specific primers (Table 1, column 4) were used in the multiplexed PCR reaction, only the 322 bp fragment was formed, as the electropherogram in Fig. 5A depicts. This suggests, that the disease was caused by the mutation of the 21-steroid hydroxylase gene (CYP 21) in the position of 1004. In a control experiment, three primers complemen-

Table 1
Tested mutation sites on the CYP-21 gene with the corresponding mutations and the normal (wild type) and mutant allele specific primers

Mutation Site (position)	Mutation (Wt→Mt)	Normal (Wild Type) Primer	Mutant Primer
1004	T→A	CCG AAG GTG AGG TAA CAG A	CGA AGG TGA GGT AAC AGT
1388	T→A	GCC TCA GCT GCA TCT CCA	GCC TCA GCT GCA TCT CCT
1999	C→T	TGG TCT AGC TCC TCC TG	TGG TCT AGC TCC TCC TA
Antisense primer		CCT GTC CTT GGG AGA CTA CT	

All sequences are given in a 5′–3′ direction.

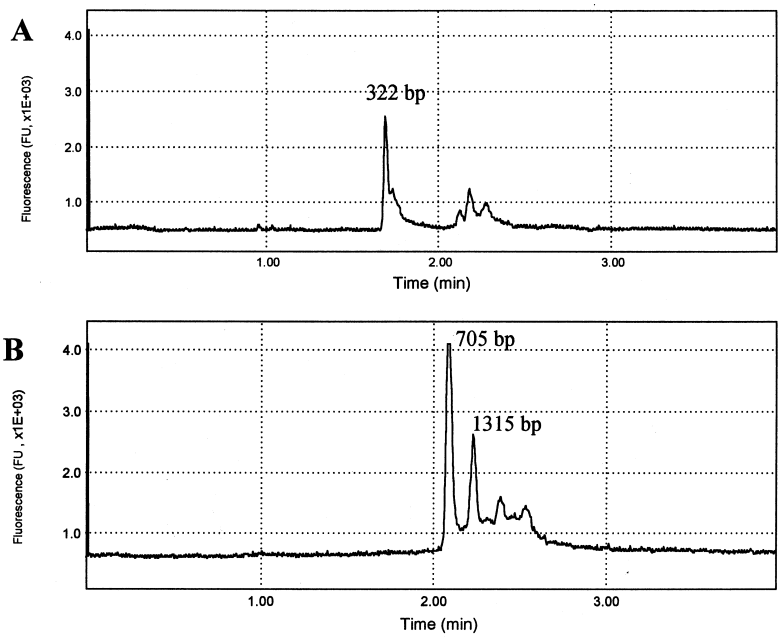

Fig. 5. Screening for mutations by multi-allele specific amplification (MASA) and capillary gel electrophoresis (CGE). A: Mutant primers (Table 1) were used as sense primers, annealing to the DNA only if any mutation was present in the gene investigated. B: Normal (wild type) primers (Table 1) were used as sense primers, resulting PCR products only in the absence of the mutations in the gene of interest. PCR reactions were made in the presence of a common antisense primer and three different sense primers (Mutant: A, Normal: B), other conditions were as described under Section 2. Aliquots of the PCR products were analyzed by CGE–LIF. Conditions of separation were the same as in Fig. 3.

tary with the normal (wild type) DNA sequence (Table 1, column 3) were also applied. In this instance, as the separation in Fig. 5B delineates, two fragments were formed, with chain lengths of 705 and 1315 bp, respectively. The simple fact, that in Fig. 5B the 322 bp fragment is missing, suggests that this patient has only one mutated form in both of the alleles, apparently causing the disease. Please note, that the comparison of the two multiplex PCR reactions clearly depicts the mutation in both the alleles at position 1004, and no mutation in positions 1388 and 1999. The small peaks at the end of both electropherograms are remaining fragments from the first, genomic PCR reaction.

4. Conclusion

Multiple mutations on the 21-hydroxylase gene (CYP-21) were investigated by multi-allele specific amplification and capillary gel electrophoresis. The genetic basis of the 21-hydroxylase enzyme deficiency was proven by using multiplexed allele specific primers in a single PCR reaction. In this way, several allele specific fragments were obtained, each with various lengths, and consequently separated and analyzed by CGE–LIF. Based on the principle of multi-allele specific amplification and the high resolution power of capillary gel electrophoresis, other known mutations could be tested on any genes by the method described above.

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References

[1] K.E. Davies, Human Genetic Disease Analysis, Oxford University Press, Oxford, 2nd ed., 1993.
[2] M.I. New, J. Steroid Biochem. Molec. Biol. 48 (1994) 15.
[3] D. Owerbach, M.B. Draznin, R.J. Carpenter, F. Greenberg, Hum. Genet. 89 (1992) 109.
[4] R. Newton, A. Graham, L.E. Heptinstall, S.J. Powell, C. Summers, N. Kalsheker, J.C. Smidt, A.F. Markham, Nucleic Acid Res. 17 (1989) 2503.
[5] D.N. Heiger, A.S. Cohen, B.L. Karger, J. Chromatogr. 516 (1990) 33.
[6] P.D. Grossman, D.S. Soane, J. Chromatogr. 559 (1991) 257.
[7] A. Guttman, B. Wanders, N. Cooke, Anal. Chem. 64 (1992) 2348.
[8] K. Ulfelder, H.E. Schwartz, J.M. Hall, F.J. Sunzeri, Anal. Biochem. 200 (1992) 260.
[9] M. Chiari, M. Nesi, P.G. Righetti, Electrophoresis 15 (1994) 616.
[10] A.E. Barron, W.M. Sunada, H.W. Blanch, Electrophoresis 16 (1995) 64.
[11] Y.F. Cheng, N.J. Dovichi, Science 242 (1989) 562.
[12] A. Guttman, N. Cooke, Anal. Chem. 63 (1991) 2038.
[13] R.P. Haugland, Nucleic acid stains, in: K. Larison (Ed.), Molecular Probes: Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes, Eugene, OR, 1992, p. 221.
[14] K. Nemeth, G. Fekete, E. Kiss, A. Varadi, K. Holics, R. Ujhelyi, J. Inherit. Met. Dis. 19 (1996) 378.
[15] A. Wedell, H. Luthman, Hum. Genet. 91 (1993) 236.